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EBNA-2 and EBNA-LP cooperate to cause G_0 to G_1 transition during immortalization of resting human B lymphocytes by Epstein–Barr virus

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Epstein–Barr virus (EBV) is unusual among DNA tumour viruses in that the virus particle is able to infect and immortalize resting cells with very high efficiency. Mutation of the viral genome has indicated that at least six viral genes (LMP-1 and EBNAs 1, 2, 3A, 3C and LP) are essential for immortalization. We demonstrate that the activation of a G_1 cyclin, cyclin D2, is an early event following infection with EBV and that cyclin D2 activation is dependent on the expression of viral genes. The different levels of cyclin D2 transcripts in Burkitt's lymphoma cell lines expressing different subsets of EBV immortalizing genes suggest an involvement of EBNA-2 or EBNA-LP in cyclin D2 regulation. By exposing resting primary B cells to a purified preparation of the EBV surface glycoprotein gp340, we have been able to achieve efficient expression of plasmid DNAs introduced by electroporation. Vectors encoding two viral genes, EBNA-2 and EBNA-LP, are sufficient to activate the expression of cyclin D2 in this system. Thus, the progression of resting B lymphocytes into the G_1 phase of the cell cycle can be reconstituted in the absence of virus by the cooperation of two of the six viral genes required for immortalization.

Key words: B lymphocyte/cyclin D/EBNA/EBV/immortalization

Introduction

Epstein–Barr virus (EBV) is a human herpes virus with which at least 90% of the world's population are infected. EBV is extremely efficient in immortalizing human B lymphocytes, and permanent lymphoblastoid cell lines (LCLs) are readily obtained by infecting resting human peripheral B lymphocytes with the virus. In normal EBV-infected individuals a stasis is established consistent with immune surveillance of EBV-immortalized B lymphocytes; however, in immunosuppressed individuals, including AIDS patients, this surveillance may fail and immunoblastic lymphomas can result. EBV is also found in the tumour cells of endemic Burkitt's lymphoma (BL), in the Reed–Sternberg cells of many cases of Hodgkin's lymphoma and in the tumour cells of nasopharyngeal carcinoma, indicating that it may be involved in the aetiology of these neoplastic disorders (Liebowitz and Kieff, 1993).

Although the EBV genome is currently known to contain >80 genes, only 11 of these are expressed in established LCLs, suggesting that the genes responsible for immortalization are within this subset (reviewed in Liebowitz and Kieff, 1993). Mutagenesis of the viral genome has further refined this subset to six genes, five of which (EBNA-1, -2, -3A, -3C and LMP-1) are absolutely required for immortalization, whereas mutation of EBNA-LP results in a low efficiency of immortalization that is initially feeder cell-dependent (Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991; Allan *et al.*, 1992). Several groups have tried to establish the order in which these genes operate by monitoring the time course of their expression during the immortalization process (Allday *et al.*, 1989; Rooney *et al.*, 1989a; Alfieri *et al.*, 1991). Although based on purified B lymphocytes from different sources, all of these studies indicate that the first EBV genes to be expressed are EBNA-2 and EBNA-LP, followed by the remaining EBNAs and LMP-1, and then the EBER1 and EBER2 RNAs.

The implicit assumption is that the products of these six viral genes regulate the expression or function of specific cell genes that participate in growth control. It is therefore central to the understanding of EBV-induced immortalization to identify such target genes, but relatively few candidates have come to light so far. Since the immortalization of a resting B lymphocyte is likely to involve the same mechanisms that enable a cell to progress through a division cycle, it is tempting to speculate that the timing of EBV latent gene expression is somehow linked to the distinct transitions involved in this process. In mammalian cells, transit through the cell cycle is thought to be regulated by the action of specific protein kinase complexes, each comprising a cyclin and its associated cyclin-dependent kinase (cdk) subunit (Sherr, 1993). For example, the complex of cyclin E and cdk2 appears to regulate the G_1/S transition, cyclin A/cdk2 functions in S and G_2, and the classic cyclin B regulates mitosis through its association with cdc2 (reviewed in Motokura, 1993; Nigg, 1993; Pines, 1993). Similarly, although the complexes between cyclins D1, D2 and D3 and their kinase partners cdk4 and cdk6 are less well understood, they are generally assumed to function during the G_1 phase (Pines, 1993).

Deregulated expression of D cyclins has been implicated in lymphomagenesis in both B and T lineages (Rosenberg *et al.*, 1991; Withers *et al.*, 1991; Lammie *et al.*, 1992; Seto *et al.*, 1992; Hanna *et al.*, 1993), and in a previous survey of human B lymphomas we noted an intriguing correlation between the presence of cyclin D2 transcripts and the expression of the full set of EBV immortalizing

genes (Palmero *et al.*, 1993). The data suggested that cyclin D2 might be a relevant target for regulation by EBV during the first stage of immortalization. Here we establish that the activation of cyclin D2 expression is an early event following infection of B lymphocytes with EBV and that it requires both protein synthesis and an intact viral genome, suggesting a requirement for the expression of viral genes.

To examine which of the viral genes was responsible for activating cyclin D2, it was necessary to develop a novel procedure that allows efficient expression of exogenous DNA in resting lymphocytes. In the normal course of EBV infection, it appears that the binding of the virus to its cell surface receptor, CD21, plays two critical roles: (i) it facilitates entry of the virus (reviewed in Liebowitz and Kieff, 1993) and (ii) it apparently triggers signals that allow viral gene expression in the resting cell (Gordon *et al.*, 1986; Hurley and Thorley-Lawson, 1989). By exposing resting B lymphocytes to purified gp340 (Madej *et al.*, 1992), a viral glycoprotein that interacts with CD21 (Tanner *et al.*, 1987), we succeeded in achieving expression from transfected plasmid DNAs. Significantly, plasmids encoding EBNA-LP and EBNA-2 can together activate the expression of cyclin D2 in the absence of other viral genes, but neither does so individually. Thus, because cyclin D2 can be regarded as a marker for the G_1 phase of the cell cycle, only two of the viral immortalizing genes are required for the progression of a resting B lymphocyte from G_0 into G_1.

Results

Cell-cycle progression in EBV-infected B lymphocytes

Human B lymphocytes were purified from pooled buffy coat preparations from peripheral blood by centrifugation over Ficoll gradients followed by positive selection on CD19 immunobeads. After release from the beads, >94% of the cells were positive for CD19 and CD37, antigens that are characteristic for B lymphocytes (data not shown). FACS analysis of propidium iodide-stained nuclei and the negligible incorporation of [^{3}H]thymidine, typically 500-fold less than in an immortalized LCL, confirmed that the cells were not actively cycling (Figure 1A and other data not shown). When such purified B lymphocytes were infected with the B95-8 strain of EBV, at least 35% of the cells showed positive staining for EBNA-2 at 2 days post-infection (data not shown). A proportion of the cells also entered the division cycle as judged by [^{3}H]thymidine incorporation, which began between 48 and 72 h post-infection (Figure 1A). Neither uninfected cells nor cells infected with UV-inactivated virus synthesized DNA over this time period (Figure 1A). That the [^{3}H]thymidine incorporation reflected entry into S phase, and not DNA repair or viral replication, was confirmed by the expression of cyclin E and proliferating cell nuclear antigen (PCNA), two well-established cell-cycle markers (Figure 1B).

The infected B lymphocytes also showed the expected temporal order of appearance of immortalizing gene products over this time period. For example, high levels of the EBNA-LP family of proteins were detected as early as 24 h post-infection, whereas EBNA-2 levels were relatively low at this stage, as judged by immunoblotting (Figure 1B). EBNA-2 subsequently accumulated to reach maximum levels by 72 h, but LMP-1, whose expression can be dependent on transactivation by EBNA-2 (Cordier *et al.*, 1990; Fahraeus *et al.*, 1990; Wang *et al.*, 1990), was not readily detectable until 144 h post-infection (Figure 1B). These findings were entirely consistent with previous reports (Allday *et al.*, 1989; Rooney *et al.*, 1989a; Alfieri *et al.*, 1991) and confirmed that our purified B lymphocyte preparations were susceptible to infection and immortalization by EBV.

Cyclin D2 expression in EBV-immortalized B lymphocytes

We have suggested previously that cyclin D2 may be a target gene for EBV (Palmero *et al.*, 1993). To assess whether the expression of cyclin D2 is regulated in response to immortalization by EBV, the levels of cyclin D2 RNA were compared in primary B lymphocytes, the recently immortalized LCL#3 cells, established LCLs and the group III BL cell line Jijoye. An ~650 bp *Nco*I–*Eco*RI restriction enzyme fragment from the human cyclin D2 gene, spanning the splice donor site of exon I (Inaba *et al.*, 1992; Xiong *et al.*, 1992), was used as a probe for RNase protection assays (Figure 2A). The integrity of each RNA was assessed with an antisense probe for 18S rRNA; no RNase-resistant products were detected with control yeast RNA. Under these conditions, no cyclin D2 RNA was detected in primary B lymphocytes, whereas immortalized LCLs and group III BL cells expressed relatively high levels (Figure 2B), as reported previously (Palmero *et al.*, 1993). In the absence of a detectable signal in primary cells it is impossible to quantitate the degree of induction, but as argued later we believe that the difference reflects the activation of an otherwise silent gene. This dramatic difference in RNA levels was also reflected in the synthesis of cyclin D2 protein in an LCL compared with primary B lymphocytes, as revealed by metabolic labelling and immunoprecipitation with a monoclonal antibody against human cyclin D2 (Figure 2C).

Dependence of cyclin D2 induction on protein synthesis and viral gene expression in EBV-infected lymphocytes

Having established a possible link between cyclin D2 expression and the events leading to immortalization by EBV, we analysed the time course of this induction following infection of purified B cells with EBV. Quantitative RNase protection assays indicated that cyclin D2 RNA was undetectable at 12 h (Figure 3A) but had reached maximum levels by 24 h post-infection (Figure 3A–C). Cyclin D2 expression therefore precedes the onset of DNA synthesis by a considerable margin (Figure 1A) and correlates more closely with the initial detection of EBNA-2; EBNA-LP is already present at high levels by this time point (Figure 1B).

The delayed induction of cyclin D2, following infection by EBV, suggested that it might require novel protein synthesis. This was assessed by treating EBV-infected B cells with the protein synthesis inhibitor anisomycin. Under the conditions used, the incorporation of [^{35}S]methionine into cell protein was decreased by at least 95% and there was no detectable expression of EBNA-2 in the infected cells as judged by immunoblotting (data

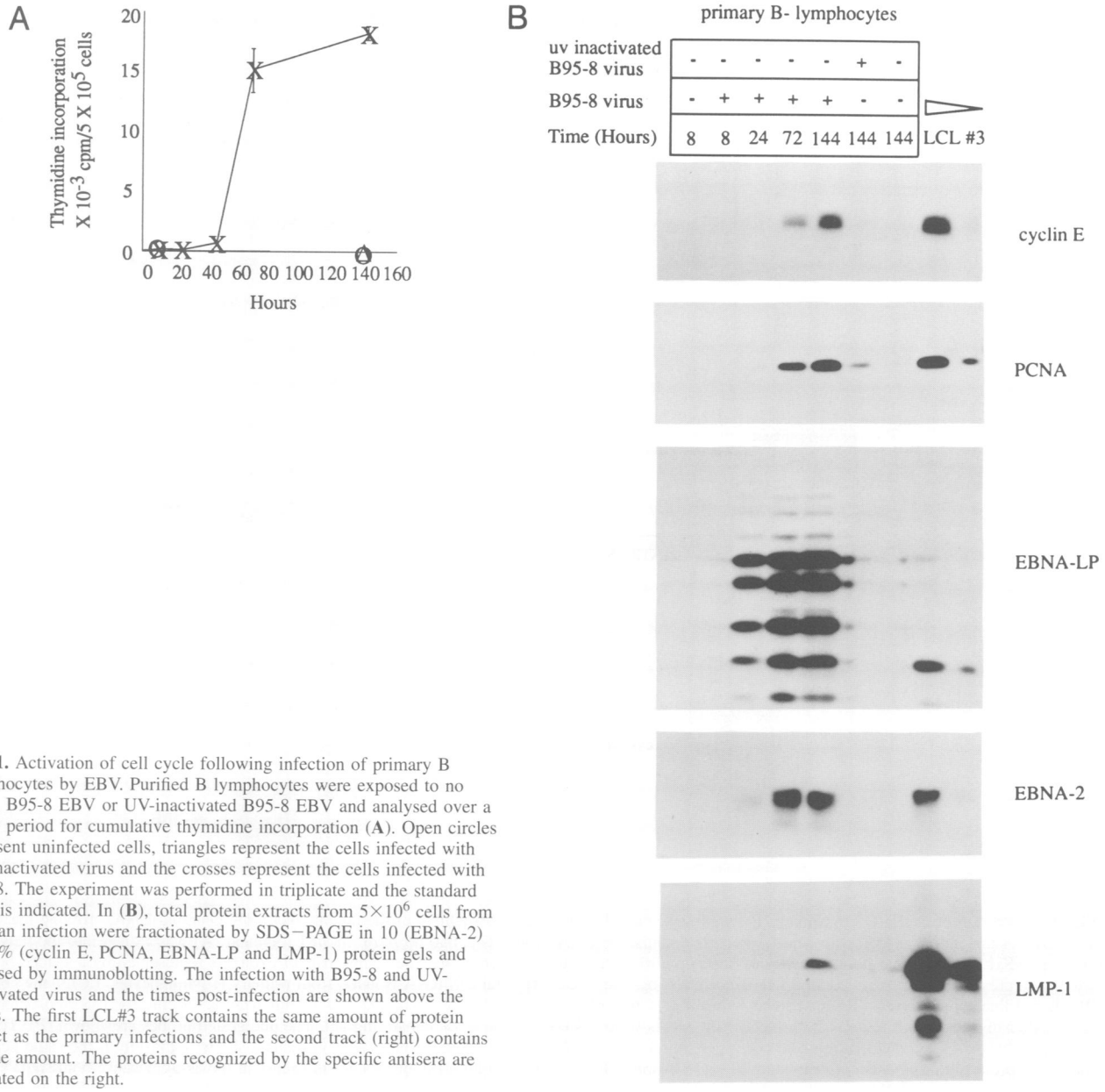

Fig. 1. Activation of cell cycle following infection of primary B lymphocytes by EBV. Purified B lymphocytes were exposed to no virus, B95-8 EBV or UV-inactivated B95-8 EBV and analysed over a 6 day period for cumulative thymidine incorporation (**A**). Open circles represent uninfected cells, triangles represent the cells infected with UV-inactivated virus and the crosses represent the cells infected with B95-8. The experiment was performed in triplicate and the standard error is indicated. In (**B**), total protein extracts from 5×10^6 cells from such an infection were fractionated by SDS–PAGE in 10 (EBNA-2) or 15% (cyclin E, PCNA, EBNA-LP and LMP-1) protein gels and analysed by immunoblotting. The infection with B95-8 and UV-inactivated virus and the times post-infection are shown above the tracks. The first LCL#3 track contains the same amount of protein extract as the primary infections and the second track (right) contains 1/5 the amount. The proteins recognized by the specific antisera are indicated on the right.

not shown). Treatment with anisomycin also prevented the activation of cyclin D2 RNA expression (Figure 3B), confirming the dependence on protein synthesis. However, as these data did not differentiate between cell or viral proteins, the contribution of the viral genome was assessed by exposure to UV-irradiation prior to infection. UV-inactivation of the virus prevented both viral gene expression (data not shown) and the accumulation of cyclin D2 RNA at 24 and 48 h post-infection (Figure 3C). These data clearly implicate protein synthesis and viral genes in up-regulating cyclin D2 expression during immortalization by EBV.

Correlation of cyclin D2 RNA levels with EBNA-2 and EBNA-LP expression in BL cell lines

Among the viral genes that may influence cyclin D2 expression in EBV-infected cells, the most likely candidates are EBNA-LP and EBNA-2, based on the timing of expression. Significantly, the same conclusion was drawn from analysing cyclin D2 expression in BL cell lines. As reported previously (Palmero *et al.*, 1993), the 7.0 and 1.7 kb cyclin D2 transcripts were readily detected in cells that express the full subset of EBV immortalizing genes, including LCLs, such as BM 16 and Bristol 8, and group III BL cell lines such as Jijoye (Figure 4). However, when this survey was extended to two additional BL lines, Daudi and P3HR1, both of which have suffered deletions within the viral genome that disrupt the coding regions for EBNA-2 and EBNA-LP, we found no detectable cyclin D2 RNA (Figure 4). Thus, two independent lines of evidence show that the expression of EBNA-2 and EBNA-LP correlates with the expression of cyclin D2.

Priming of resting B lymphocytes with EBV gp340

To try to substantiate these correlations, we sought a means of introducing and expressing individual viral genes in resting B lymphocytes. However, it has been shown previously that only ~2% of primary B lymphocytes

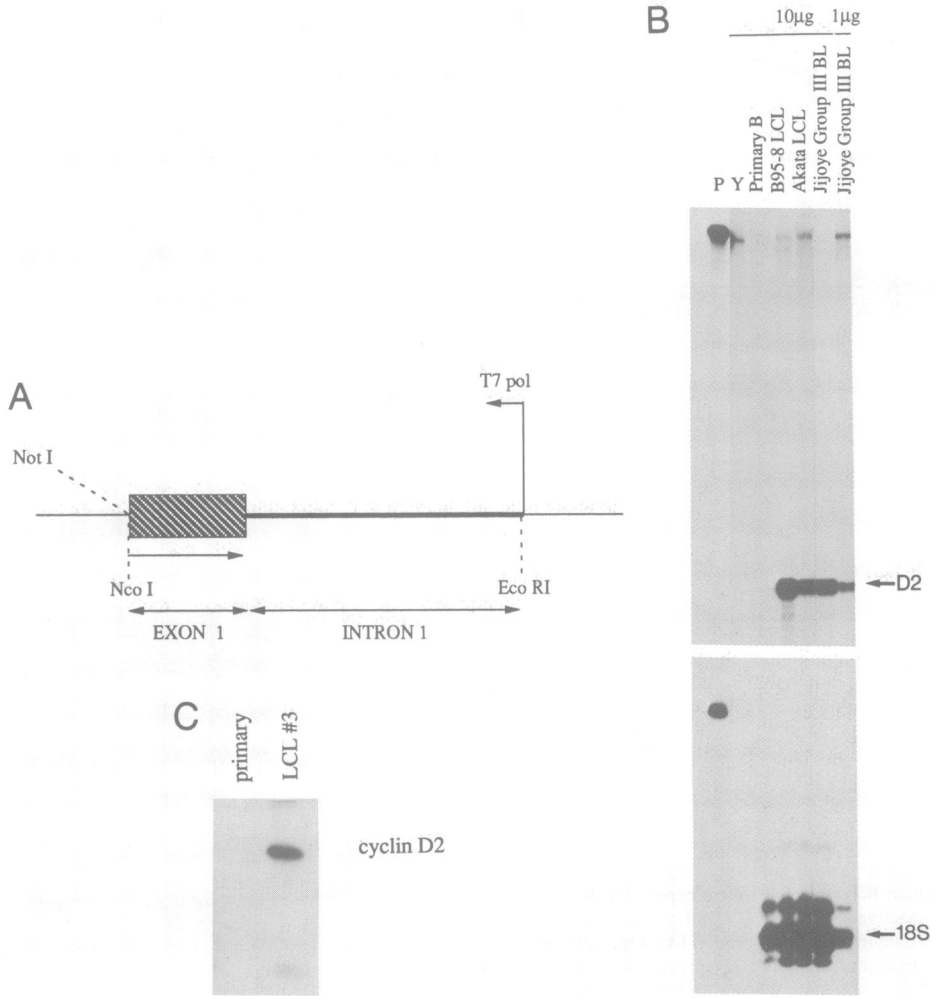

Fig. 2. Expression of cyclin D2 in primary B lymphocytes and LCL#3 cells. A fragment of the human cyclin D2 genomic DNA, crossing the splice donor site at the end of exon I, was used to prepare an antisense RNA probe. This 650 bp *Nco*I−*Eco*RI fragment (**A**) was cloned into the polylinker of the pBSKII vector so that it could be transcribed *in vitro* with T7 RNA polymerase. The coding part of exon I is represented by a shaded box, the intron is represented by a thick line and the vector DNA by thin lines. (**B**) Total cell RNA isolated from primary B lymphocytes, LCLs, the group III BL cell line Jijoye or yeast RNA (Y) was hybridized with a riboprobe specific for cyclin D2 and a similar probe specific for 18S ribosomal RNA. After digestion with RNase, the protected fragments were fractionated on a 6% denaturing gel. The undigested probes are shown (P). The upper panel shows the protected fragment corresponding to cyclin D2 (arrowed). The lower panel represents a shorter exposure of the same gel and shows the protected fragments corresponding to 18S ribosomal RNA. Note that both riboprobes are in excess in the assay because both signals are reduced when less input RNA is used (right lane). (**C**) Purified primary B lymphocytes and an LCL (LCL#3) were labelled with [^{35}S]methionine, and cyclin D2 protein was detected by immunoprecipitation and SDS−PAGE in a 17.5% gel.

support the expression of transfected DNA without prior stimulation (Pilon *et al.*, 1991; Peng and Lundgren, 1992). This efficiency can be increased by prior stimulation with TPA, suggesting that the accumulation of some endogenous gene products facilitates transcription of the input DNA. As an example of this effect, the expression of a chloramphenicol acetyl transferase (CAT) marker gene under the control of the immediate early promoter of cytomegalovirus (CMV) could be activated by culturing the resting lymphocytes in TPA (Figure 5A). However, treatment with TPA was itself sufficient to induce expression of cyclin D2 in the absence of viral infection (Figure 5B) and the cells progressed into S phase as judged by [^{3}H]thymidine incorporation (data not shown).

Since the EBV genome is clearly expressed in resting B lymphocytes, the virus must have some way of activating the machinery required to transcribe viral genes. We therefore reasoned that by mimicking some aspect of viral entry it may be possible to achieve expression of transfected plasmids. For example, infection by EBV is accompanied by changes in surface CD23 expression and chromosome accessibility that are independent of viral gene expression and may simply require attachment or entry of the viral particle (Gordon *et al.*, 1986; Hurley and Thorley-Lawson, 1989). The binding of EBV to B lymphocytes is mediated by interaction between the N-terminus of a viral glycoprotein, gp340, and the cell surface receptor CD21 (Nemerow *et al.*, 1989). To achieve the same effect, resting B lymphocytes were exposed to purified gp340, lacking its membrane anchor region (Madej *et al.*, 1992), immobilized on the surface of tissue culture dishes. By priming the cells in this way before electroporation, expression of the CMV-CAT marker gene was increased 25-fold (Figure 5A). It is not yet known whether the priming increases the per cent of transfected cells, the amount of DNA taken up, or both. However, the expression

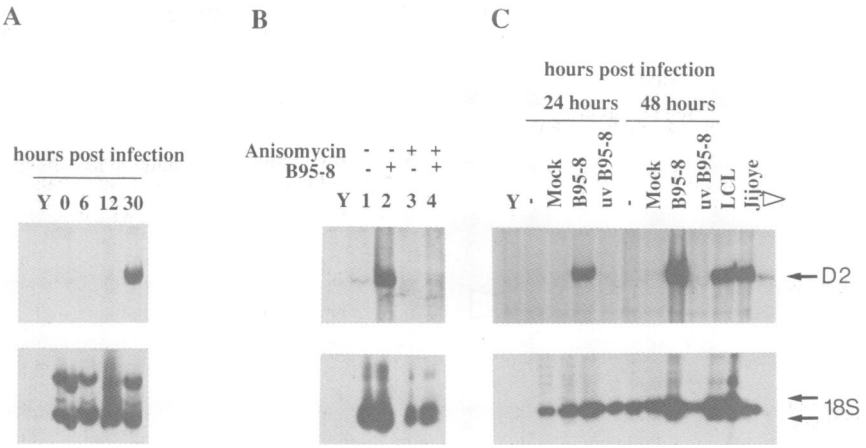

Fig. 3. Cyclin D2 RNA expression following infection of B lymphocytes with B95-8 EBV. (**A**) Purified B lymphocytes were infected with B95-8 EBV and the levels of cyclin D2 RNA and 18S ribosomal RNA were analysed by RNase protection assay between 0 and 30 h post-infection. (**B**) Purified B lymphocytes were incubated for 6 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the protein synthesis inhibitor anisomycin and infected with B95-8 EBV where indicated (lanes 2 and 4). The levels of cyclin D2 RNA and 18S ribosomal RNA were analysed by RNase protection assay 24 h post-infection. (**C**) Purified B lymphocytes were infected with B95-8 EBV, UV-inactivated B95-8 EBV or mock infected. The levels of cyclin D2 RNA and 18S ribosomal RNA were analysed by RNase protection assay 24 and 48 h post-infection. The upper panels show the signals corresponding to cyclin D2 and the lower panels show the signals corresponding to 18S rRNA. Controls include hybridization to yeast RNA (Y) and RNA from cell lines LCL#3 and Jijoye cells, both of which express cyclin D2. The analysis of 10-fold less Jijoye RNA (right lane) confirms that the assay was performed in probe excess.

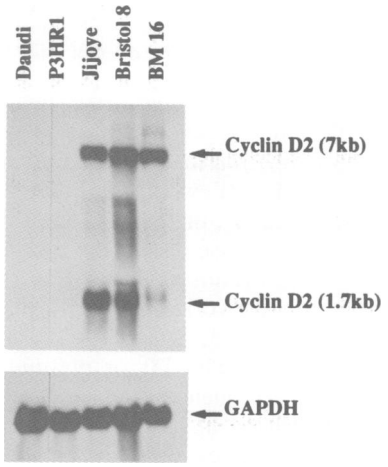

Fig. 4. Expression of cyclin D2 RNA in BL cell lines. Total cell RNA (10 μg) from the indicated cell lines was fractionated on a formaldehyde-agarose gel and analysed by blot hybridization as described (Palmero *et al.*, 1993). Daudi and P3HR1 are BL cell lines with deletions within the EBNA-2 and EBNA-LP coding regions of EBV; Jijoye is a group III BL containing wild type EBV; Bristol 8 and BM 16 are LCLs. The signals corresponding to the two RNA species encoding cyclin D2 are indicated in the upper panel and the signal corresponding to GAPDH is in the lower panel.

of cyclin D2 was not activated by this treatment (Figure 5B) and the cells did not enter S phase (data not shown). By pretreating resting B lymphocytes with gp340, we could therefore achieve efficient expression of exogenous DNA without causing the cells to enter the cell cycle.

Activation of cyclin D2 expression by EBNA-2 and EBNA-LP

The ability to transfect resting B lymphocytes after priming with gp340 enabled us to examine directly the role of EBNA-LP and EBNA-2 in the induction of cyclin D2 expression. However, because only a small number of cells could be transfected in this way, a more sensitive assay system for cyclin D2 RNA based on reverse-transcription and amplification by the polymerase chain reaction (RT-PCR) was devised. PCR primers spanning exons 1 and 2 of human cyclin D2 gave a readily detectable 170 bp product using RNA from an LCL. Primers for the 36B4 gene (Rich and Steitz, 1987) served as a control. In a reconstruction experiment (not shown), we have demonstrated that this assay is capable of detecting cyclin D2 transcripts at levels that are 10^5-fold lower than in an LCL. Significantly, no signal was detected in primary B lymphocytes using this assay, suggesting that the cyclin D2 gene may be transcriptionally silent. When plasmid vectors encoding either EBNA-LP or EBNA-2 were introduced into gp340-primed B lymphocytes by electroporation, cyclin D2 remained undetectable (Figure 6). However, when the two plasmids were introduced together, a clear cyclin D2 signal was observed (Figure 6). RT-PCR of dilutions of this RNA showed that this represented at least a 25-fold induction of cyclin D2 RNA in response to cotransfection of plasmids expressing EBNA-2 and EBNA-LP (data not shown). In other experiments we have confirmed that the transfection of these plasmids into B lymphoma cells supports the expression of the respective protein and that the level of one is not affected by cotransfection of the other plasmid. The data in Figure 6 therefore indicate that the concerted action of these two viral genes, EBNA-LP and EBNA-2, is sufficient to cause resting B cells primed with gp340 to enter G_1 and to express cyclin D2.

Discussion

The experiments we describe address a critical phase in the immortalization of human B lymphocytes by EBV, a process that is likely to be relevant to the development of the lymphoblastic disorders associated with EBV infection,

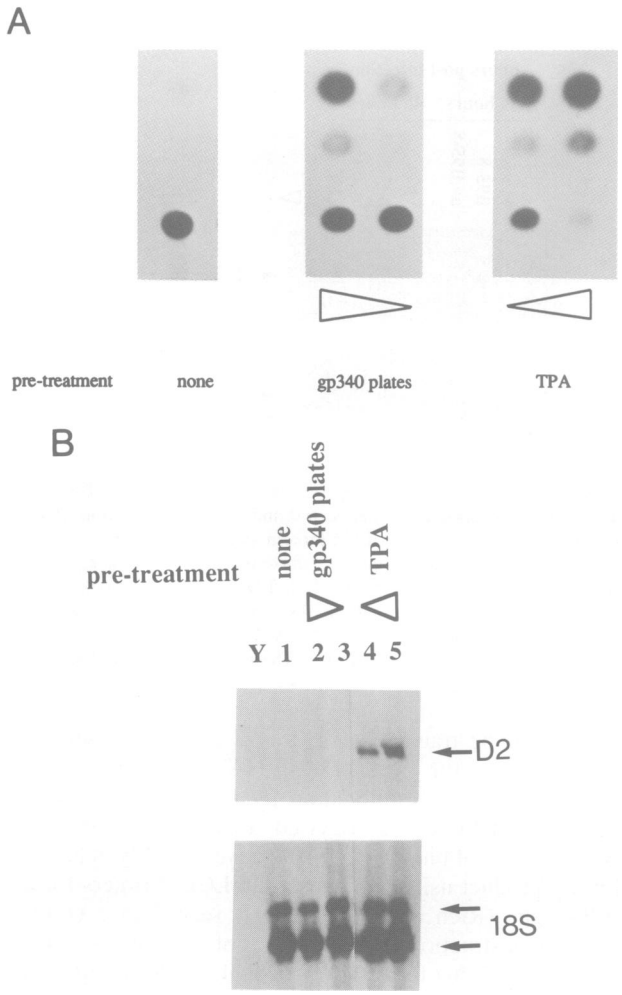

Fig. 5. Reporter gene expression in gp340-primed B lymphocytes. (**A**) Purified B lymphocytes were pretreated for 20 h with gp340 (4 or 40 ng/ml) or TPA (4 or 40 ng/ml) and then electroporated with pCMVIE-CAT. After 24 h the CAT activity was determined by thin layer chromatography. (**B**) Samples of RNA extracted from lymphocytes pretreated as in (A) were assayed for cyclin D2 RNA and 18S ribosomal RNA by quantitative RNase protection assay. Track Y is the yeast RNA negative control.

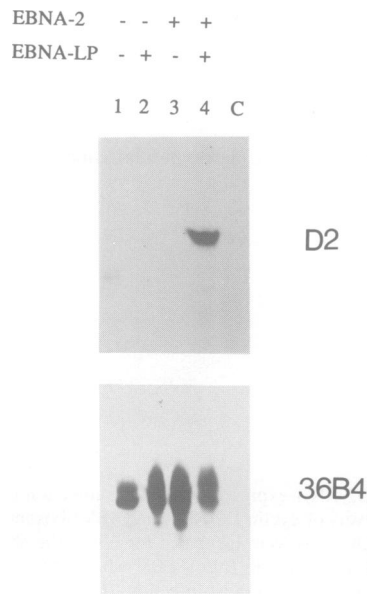

Fig. 6. Co-expression of EBNA-LP and EBNA-2 induces cyclin D2 expression in gp340-primed B lymphocytes. Purified B lymphocytes were preincubated in plastic dishes coated with 40 ng/ml gp340 for 20 h prior to electroporation with vectors p3.1W-LP and pEΔA6 expressing EBNA-LP or EBNA-2. RNA was harvested after 24 h and assayed for expression of cyclin D2 and the ribosomal protein 36B4 by RT-PCR. Track C contains a control reaction using yeast RNA.

and possibly BL. Uniquely among the known DNA tumour viruses, EBV can very efficiently convert a truly resting B lymphocyte into an immortal cell line (Liebowitz and Kieff, 1993). The purified B lymphocytes used in this study were evidently in G_0, as they showed negligible [^{3}H]thymidine incorporation (Figure 1A) and were unable to support expression of exogenous DNA introduced by electroporation (Figure 5A). Upon infection by EBV, the cells synchronously entered the cell cycle, with DNA synthesis beginning at ~48 h accompanied by increased expression of cyclin E and PCNA, two established markers for G_1/S progression (Figure 1B). However, these events are preceded by the expression of cyclin D2, which to date is the earliest cell gene known to be activated by EBV infection (Figure 3A). For this reason, we have focused our attention on cyclin D2 because it provides an ideal marker for the initial stages of immortalization by EBV, the transition from G_0 into G_1.

Several lines of evidence identify cyclin D2 expression as a G_1 marker. At least one of the D cyclins, D1, is able to complement G_1 cyclin function in strains of *Saccharomyces cerevisiae* that are conditionally deficient in *CLN* gene function (Lew *et al.*, 1991; Xiong *et al.*, 1991). More significantly, the expression of the D cyclins increases in G_1 in response to specific cytokines, leading to suggestions that D cyclins might serve as a link between signal transduction networks and cell-cycle regulation (reviewed in Pines, 1993; Sherr, 1993). Microinjection of antibodies against cyclin D1 or plasmids expressing antisense cyclin D1 sequences can block entry of cells into S phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993), whereas constitutive expression of cyclin D1 or cyclin D2 can reduce the time required for progression through G_1 (Ando *et al.*, 1993; Jiang *et al.*, 1993) and overcome the G_1/S block mediated by Rb in SAOS-2 cells (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993).

This latter result is intriguing in that the D cyclins are reported to bind directly to pRb and complexes of D cyclins, and cdks, particularly cyclin D2/cdk4, are able to phosphorylate pRb, at least *in vitro* (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993). It is therefore possible that the induction of cyclin D2 is part of a mechanism by which EBV inactivates the growth suppressive functions of pRb. Other DNA tumour viruses, such as SV40, HPV16 and adenovirus, achieve the same effects through a direct interaction between viral gene products and tumour suppressor proteins (Lane and Benchimol, 1990; Nevins, 1992). It is not clear why EBV should have evolved a different and more complex mechanism for immortalization requiring several genes but it may reflect the fact that EBV infects a resting cell.

Attempts to dissect the initial events in the immortalization process have been hampered by the difficulties in achieving exogenous gene expression in the quiescent

target cells. By exposing B lymphocytes to purified preparations of gp340, we have succeeded in circumventing these problems and shown that of the six viral genes that are essential for immortalization, only two, EBNA-LP and EBNA-2, are required to bring resting B lymphocytes into the G_1 phase of the cycle, as judged by the activation of cyclin D2. The analysis of viral mutants, such as those present in P3HR1 and Daudi cells (Figure 4), and the timing of the expression of EBV genes relative to that of cyclin D2 (Figures 1 and 3) had already suggested the importance of EBNA-LP and EBNA-2, but the data presented in Figure 6 confirm that they act in concert to induce expression of cyclin D2. Presumably, the other essential gene products are required for later steps in cell-cycle progression, in line with their temporal order of appearance (Figure 1B). For example, Peng and Lundgren (1992) have reported that LMP-1, which is expressed at the G_1/S boundary following EBV infection, can increase incorporation of [^{3}H]thymidine in primary B lymphocytes pretreated with either TPA or UV-inactivated EBV. How these data relate to the current findings is uncertain because we have shown that TPA induces cyclin D2 expression (Figure 5) whereas UV-inactivated virus does not (Figure 3C). However, the system we have developed should help clarify the sequential contributions of the EBV genes in the immortalization of B lymphocytes.

In conclusion, the experiments reported here demonstrate that the first stage of the disruption of cell-cycle control by EBV, namely the progression from G_0 into G_1, can be achieved by the expression of only two viral genes. By defining further markers for cell-cycle progression, followed by the systematic introduction of expression vectors for each of the immortalization genes into gp340-primed B lymphocytes, it may eventually be possible to reconstruct the immortalization process in the absence of virus.

Materials and methods

Cell culture and purification of primary B lymphocytes

The Jijoye, P3HR1 and Daudi cell lines were obtained from ATCC. BM 16 and Bristol 8 were obtained from the ICRF Cell Production facility. Cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin and 10% heat-inactivated fetal calf serum at a density of 2–5×10^5 cells/ml. LCL#3 was generated by immortalization of purified primary B lymphocytes with the B95-8 strain of EBV and this line had been in culture for <6 months at the time of this investigation.

Primary B lymphocytes were purified from human peripheral blood purchased from North London Blood Transfusion Service. Buffy coats were subjected to centrifugation over Ficoll paque gradients (Pharmacia) and the CD19-positive lymphocytes were immunoselected with pan-B Dynal M450 beads (Funderud *et al.*, 1990). After release from the beads with Detachabead (Dynal), the cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin and 15% heat-inactivated fetal calf serum at a density of 10^6 cells/ml for 16 h prior to use.

Purification of B95-8 EBV virus

EBV was purified from B95-8 cells treated with 30 ng/ml TPA for 6 days. Following centrifugation at 1300 r.p.m. for 5 min and filtration of the medium through a 0.45 μm filter (Nalgene), the virus was pelleted by centrifugation at 27 000 r.p.m. for 60 min. The pellet was washed carefully to remove any traces of TPA, resuspended and stored at −80°C. Where indicated, virus stocks were inactivated by pretreatment with 9 mJ of UV radiation with a Stratalinker (Stratagene).

Thymidine incorporation assays

Samples of 10^6 cells were incubated in medium containing 1 μCi of [^{3}H]thymidine and harvested at various times, typically 8, 24, 48, 72 and 144 h after infection by EBV. The cells were lysed in 100 μl of water, precipitated with 10% TCA and the precipitated material collected on a GF/C filter (Whatman). Assays were performed in triplicate and the incorporation of [^{3}H]thymidine was quantitated by liquid scintillation counting.

Metabolic labelling and protein analysis

Purified B lymphocytes and LCL#3 cells were labelled with 100 μCi/ml [^{35}S]methionine (Amersham plc) for 60 min. Cells were lysed in NP-40 lysis buffer (Harlow and Lane, 1988) and adjusted for total protein concentration. Extracts from the equivalent of 5×10^7 B lymphocytes were pre-cleared with protein A−Sepharose beads (Pharmacia) and incubated with 5 μg of antibody for 60 min (G132-43; Pharmingen). The antibody complexes were isolated using rabbit anti-mouse (Z259; Dako)-coated protein A−Sepharose beads, and fractionated on a 17.5% SDS−polyacrylamide gel (Harlow and Lane, 1988).

For immunoblotting, cell lysates were fractionated directly by SDS−PAGE, transferred to membranes and developed with monoclonal antibodies for PCNA (Oncogene Science), EBNA-LP (JF186; Finke *et al.*, 1987), EBNA-2 (PE-2; Dako) and LMP-1 (CS1-4; Dako). The specific signals were visualized using a second layer of rabbit anti-mouse Ig Z259 (Dako) followed by incubation with [^{125}I]protein A (Amersham) and autoradiography.

RNase protection assays and Northern blotting

Total RNA was isolated and RNase protection assays were performed essentially as described (Sinclair *et al.*, 1991). An ~650 bp *Nco*I−*Eco*RI restriction enzyme fragment containing part of exon I and intron I of human cyclin D2 (Inaba *et al.*, 1992; Xiong *et al.*, 1992; and unpublished observations) was subcloned into the *Bam*HI and *Eco*RI sites in the BSKII vector (Stratagene). [^{32}P]UTP-labelled antisense riboprobes were prepared for cyclin D2 and for 18S ribosomal RNA (Ambion) by run-off transcription with T7 polymerase. The 18S rRNA probe was prepared at 1500-fold lower specific activity by isotopic dilution of the [^{32}P]UTP. The total cell RNA was annealed with the cyclin D2 and 18S rRNA riboprobes and specific transcripts identified by an RNase protection assay (Ambion).

For Northern blotting, samples of total cell RNA (10 μg) were fractionated on a denaturing agarose gel, transferred to nylon filters and hybridized with cyclin D2 or GAPDH-specific probes, as described previously (Palmero *et al.*, 1993).

Plasmid constructs and electroporation of primary B lymphocytes

Primary B lymphocytes were pretreated for 20 h by incubation with TPA (4 or 40 ng/ml) or in tissue culture flasks (Falcon) which had been coated previously with gp340 (4 or 40 ng/ml in PBS). Highly purified gp340 lacking the membrane anchor was the kind gift of J.Arrand and M.Mackett (Madej *et al.*, 1992). Each vector DNA (10 μg) was introduced into 1×10^7 pretreated B lymphocytes by electroporation (as described by Pilon *et al.*, 1991).

The CMVIE CAT plasmid contained the chloramphenicol acetyl transferase coding sequence cloned downstream of the CMV immediate early promoter in pSP72 (Promega). p3.1W-LP was made by cloning an EBNA-LP cDNA (Speck *et al.*, 1986) between the *Xba*I and *Eco*RI sites of p3.1W (Rooney *et al.*, 1989b), and pEΔA6 contains the EBNA-2 coding region expressed from the EBV Wp promoter and SV40 enhancer (Ricksten *et al.*, 1987). The total amount of DNA and the promoter/enhancer content in each electroporation was balanced with the vectors pSVgpt (Mulligan and Berg, 1981) and p3.1W-LPrev (which is similar to p3.1W-LP but with the insert cloned in the antisense orientation). The cells were harvested after 24 h and extracts were prepared for either CAT assays (Gorman *et al.*, 1982) or RNA analysis by RT-PCR (see below).

RT-PCR assay

For analysis of cyclin D2 RNA, first-strand cDNA was prepared (Stratagene) from total cell RNA isolated from 1×10^6 transfected B lymphocytes with a primer from cyclin D2 exon 3, CAGCACCACC-AGTTCCCACTCC. The PCR (Promega) was performed with primers from exons 1 (TTCAAGTGCGTGCAGAAGGAC) and 2 (AGTTGCAG-ATGGGACTTCGGAG). For analysis of the ribosomal protein 36B4 (Rich and Steitz, 1987), first-strand cDNA was prepared with random primers (Stratagene) and the PCR performed with the two primers GGCCGAATTCTGTCTGTGGAGACGGATTACACC and GGCCGG-ATCCGACTCTTCCTTGGCTTCAACCTTAG. The conditions for the

PCR were 30 cycles at 95°C for 1 min, 50°C for 2 min and 72°C for 2 min, yielding a radioactive fragment of 170 bp for cyclin D2 and 160 bp for 36B4. The products were analysed on a 6% denaturing acrylamide gel.

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